A highly sensitive selection method for directed evolution of homing endonucleases

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ABSTRACT

Homing endonucleases are enzymes that catalyze DNA sequence specific double-strand breaks and can significantly stimulate homologous recombination at these breaks. These enzymes have great potential for applications such as gene correction in gene therapy or gene alteration in systems biology and metabolic engineering. However, homing endonucleases have a limited natural repertoire of target sequences, which severely hamper their applications. Here we report the development of a highly sensitive selection method for the directed evolution of homing endonucleases that couples enzymatic DNA cleavage with the survival of host cells. Using I-SceI as a model homing endonuclease, we have demonstrated that cells with wild-type I-SceI showed a high cell survival rate of 80–100% in the presence of the original I-SceI recognition site, whereas cells without I-SceI showed a survival rate <0.003%. This system should also be readily applicable for directed evolution of other DNA cleavage enzymes.

INTRODUCTION

Homing endonuclease are highly specific DNA endonulceases, encoded within mobile introns or inteins (1). They recognize specific 14–40 bp DNA sequences and catalyze site-specific double-strand breaks (DSBs) in the chromosome. Homing endonucleases have great potential to be applied in gene targeting, as the site-specific DNA DSBs that they introduce can significantly stimulate homologous recombination (2). Gene correction at the mutated locus by means of homologous recombination has clear advantages over gene addition by virus-driven random integration, which suffers from the consequences of transgene silencing and improper activity. Although homologous recombination is a more attractive

method, it is presently unfeasible in most cases owing to the generally low frequency of homologous recombination. Many attempts have been made to increase the efficiency of homologous recombination in mammalian cells, such as increasing the size of the region of homology with the target locus, using isogenic genomic DNA, or improving the selection procedures (3). As a DNA DSB is lethal to cell survival, it triggers the cell repair machinery and greatly increases the frequency of homologous recombination at this site. Introduction of a DNA DSB by homing endonuclease in the gene to be replaced results in much enhanced homologous replacement by the incoming DNA, even with relatively low stretches of homologous DNA sequence (3,4).

However, the limited natural repertoire of target sequences of homing endonucleases severely hampers their application, and the lack of an efficient selection method restricts the use of powerful directed evolution approaches for engineering of homing endonucleases with altered sequence specificity *in vitro* (5). Seligman *et al.* (6) developed a positive and negative screening method for assaying homing endonuclease activity. In this system, the cleavage of the target DNA sequence by homing endonuclease results in the host cell being converted from Kan$^+$ to Kan$^-$ or from LacZ$^+$ to LacZ$^-$. Sussman *et al.* (7) successfully used this screening system to verify the target DNA specificity of homing endonuclease I-CreI variants. However, the throughput of such a system is rather limited owing to its screening nature. The *in vivo* selection system for homing endonuclease activity developed by Gruen *et al.* (8) links the catalytic activity of a homing endonuclease to the survival of an *Escherichia coli* cell through a DNA cleavage event. This system comprises two plasmids: one plasmid encodes a mutant barnase gene with two amber (TAG) stop codons under an inducible arabinose promoter, followed by tandem endonuclease cleavage sites; and the other plasmid contains a fusion protein of homing endonuclease and Amber suppressor tRNA *supE* under the constitutive *lac* promoter. The coexpression of the mutant barnase gene and the tRNA expression cassette fusion protein results in cell death. However, the cleavage of the target DNA sequence by the

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homing endonuclease before the induction of mutant barnase expression by arabinose can eliminate the plasmid encoding mutant barnase and lead to cell survival. This system can be applied to assay homing endonuclease activity *in vivo* and has higher sensitivity than the traditional *in vitro* assay. However, the drawbacks of this system include a relatively high background survival (survival of cells harboring both plasmids in the presence of arabinose, but lacking homing endonuclease activity) and a low sensitivity (cells with the wild-type homing endonuclease I-SceI showed a low cell survival rate of 25% in the presence of two tandem copies of the original I-SceI recognition site), which impedes its application in directed evolution of homing endonucleases with altered target sequence. We have developed an *in vivo* endonuclease activity assay utilizing two different plasmids that share the same principle of Gruen's system. In our system, cells with wild-type I-SceI show a high cell survival rate of 80–100% in the presence of one copy of the original I-SceI recognition site, and by incorporating a mutant LacY(A177C) as an arabinose transporter, the survival rate of cells without I-SceI is <0.003%. Such a high signal-to-background ratio makes this system suitable for application in directed evolution.

MATERIALS AND METHODS

Plasmid construction

To construct plasmid pBAD-ccdB, the *ccdB* gene was amplified from pTCKJ02 (9) (a gift from J. D. Keasling) with primer NheI-ccdB, catgca *gctagc* GGAGTG aaacgatgcagttt aaggtttacacctataaaaga (NheI site shown in italic), and ccdB-R(X+S), agtacg *gcatgc* cgtatg *tctaga* ttatattcccccaaaacatcaggttaat (XbaI and SphI sites shown in italic), and cloned into the NheI and SphI sites of pBAD18 (10) (a gift from J. M. Slauch). Primer NheI-ccdB also contains the Shine-Dalgarno (S.D.) sequence from pBAD18s (10), shown in capital letters. For ribosome binding site (RBS) engineering, this S.D. sequence was replaced by NNNNNN to form the random library and the best mutant identified from this library was named p11-ccdB. The recognition sequence of I-SceI was inserted into p11-ccdB at XbaI and SphI sites by oligos 5′-ctagc attacgc TAGGGATAACAGGGTAAT atcacgc *tctaga* catacg *gcatg*-3′ and 5′-c cgtatg *tctaga* gcgtgat ATTACCCTGTTATCCCTA gcgtaatg-3′ (recognition sequence of I-SceI shown in capital letters and XbaI and SphI sites shown in italic) to form p11-ccdB-wtx1. A modified I-SceI recognition sequence was similarly inserted into p11-ccdB by oligos 5′-ctagc attacgc TAGGGATAACAAGGTAAT atcacgc *tctaga* catacg *gcatg*-3′ and 5′-c cgtatg *tctaga* gcgtgat ATTACCTTGTTATCCCTA gcgtaatg-3′ (modified recognition sequence of I-SceI shown in capital letters and XbaI and SphI sites shown in italic) to form p11-mISceI. Ligation of these oligos into p11-ccdB XbaI and SphI sites abolished the original XbaI site and introduced a new XbaI site (shown in italic). Additional recognition sequence can be inserted into the new XbaI and SphI sites.

A synthetic cassette 5′-tacgtacga *tttaaa* taggcct-3′ was ligated into the large NdeI–ClaI fragment of p11-ccdB-wtx1 in order to introduce a StuI site. Mutant LacY(A177C) under the *lac* promoter was amplified from pLacYA177C (11) (a gift from J. E. Cronan) with primers plac-StuI, gagctc *aggcct* gactcactataggggagaccg, and LacY-StuI-R, ctagct *aggcct*

taagcgacttcattcacct (StuI sites shown in italic), and inserted into the StuI site of p11-ccdB-wtx1 to form p11-LacY-wtx1.

Plasmid pTrc99a was used as a template for I-SceI cloning. First, the ampicillin resistance gene in pTrc99a was replaced by kanamycin resistance gene to form pTrc-KM. The large fragment of pTrc99a digested with BspHI was treated with T4 DNA polymerase (New England Biolabs, Beverly, MA) to form blunt ends, and it was ligated with the blunt ended small fragment of pET26b digested with AlwNI and XhoI. Second, the pBR322 origin of replication in pTrc-KM was replaced by p15a origin to form pTrc-p15a. The large fragment of pTrc-KM from AlwNI and HpaI digestion was treated with T4 DNA polymerase to form blunt ends, and ligated with the StuI digested PCR product of pACYCDuet-1 (Novagen, Madison, WI) amplified with primers StuI-D1, tattaa*aggcct* gctccagtggcttctgtttc, and StuI-D2, ataatt *aggcct* cttgagagccttcaacccag (StuI sites shown in italic). Homing endonuclease I-SceI was amplified from pSCM525 (12) (a gift from B. Dujon) with primers EcoRI–SceI′, atcagt *gaattc* aggaaactcgagatgaaaaatattaaaaaaaa (EcoRI site shown in italic), and KpnI-Isce-2-C, atgccg *ggtacc* ttattttaaaaaagtttcgg (KpnI site shown in italic), and cloned into the KpnI and EcoRI sites of pTrc-p15a to form pTrc-ISceI. pTrc-D44A, containing a mutant I-SceI (D44A) was constructed using megaprimer PCR technique (13). A first PCR was performed with D44A′-C, gatgtaagcagcacccaggat, and EcoRI–SceI′ using pSCM525 as template to form a megaprimer. This megaprimer, in combination with KpnI-Isce-2-C, was used to amplify pSCM525 to obtain mutant I-SceI (D44A), which was then cloned into pTrc-p15a.

Ribosome binding site strength engineering

The *ccdB* gene was amplified from pTCKJ02 with primers rbs-ccdB-F, ttttgg *gctagc* nnnnnn aaacgatgcagtttaaggtttacacctataa (NheI site shown in italic), and ccdB-R(X+S), and cloned into pBAD-ccdB digested with NheI and SphI. The −6 to −12 bases of the ccdB gene were randomized with degenerate primers to form the random library of S.D. sequences. This library was transformed into BW25141 (*lacI*^{q} *rrnB*$_{T14}$ $\Delta lacZ_{WJ16}$ $\Delta phoBR580$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$ *galU95* *endA*$_{BT333}$ *uidA*(ΔMluI)::*pir*$^+$ *recA1*) (14,15) (a gift from W. W. Metcalf) and plated on Luria–Bertani (LB) plates in the presence of 100 µg/ml ampicillin. Colonies formed on the plates were transferred with sterile toothpicks into round-bottom 96-well plates (Evergreen Scientific, Los Angeles, CA) containing 50 µl LB plus 100 µg/ml ampicillin and grown overnight at 37°C with vigorous shaking. This overnight culture was then diluted with 200 µl per well sterile double-distilled water and 5 µl of this culture was transferred to the corresponding wells of two sterile flat-bottom 96-well plates (Rainin Instrument, Oakland, CA) containing 200 µl of LB plus 100 µg/ml ampicillin with or without 10 mM arabinose and incubated at 37°C for 16 h, after which OD$_{600}$ readings were taken by using a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA). Owing to the toxic nature of CcdB, it was reasoned that strong RBS would lead to little or no cell growth in the absence of arabinose induction, while weak RBS would allow high cell survival rates even at the maximum arabinose concentration. A total of 800 colonies were picked and 13 clones showing healthy

growth in LB media without arabinose and little growth in the presence of 10 mM arabinose were selected. These clones were then plated on LB plates containing 100 µg/ml ampicillin and 0, 1, 4 or 10 mM arabinose, and incubated at 37°C for 24 h. Clone p11-ccdB, which showed normal colony growth on the plate with 0 mM arabinose and no colony on plates with 4 mM arabinose, was chosen for future experiments. DNA sequencing of this clone revealed its S.D. sequence as GATTGA.

In vivo activity assay

The selection strain was made by transforming *E.coli* BW25141 with the appropriate reporter plasmid and plating on a LB plus 100 µg/ml ampicillin plate. A single colony from the transformation plate was picked and grown in 500 ml of LB plus 100 µg/ml ampicillin and electrocompetent cells of the selection strains were prepared following standard protocols (16). It is important that cells do not grow to log phase at any stage during competent cell creation. Typically, 50 µl of the competent selection strains were transformed with 1–100 ng of pTrc-ISceI/D44A or pTrc-p15a plasmids and the cultures were immediately recovered in SOC media and shaken at 37°C for 5 min. The culture was then diluted 5-fold with SOC media pre-warmed at 37°C media. For selection strains not containing the *lacY(A177C)* gene, the culture was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St Louis, MO) and shaken at 37°C for 40 min before plating on agar plates. For the selection strain containing p11-LacY-wtx1, a final concentration of 0.5 mM IPTG was used to induce protein expression from the *Trc* and *lac* promoters. This culture was allowed to grow at 37°C for 70 min followed by 1 h incubation at 30°C prior to plating on agar plates. An aliquot of cells was spread on plates of LB plus 50 µg/ml kanamycin to estimate the total number of transformants. A second aliquot was plated on LB plus 50 µg/ml kanamycin plus 10 mM arabinose plates. All plates were incubated at 37°C for 12–24 h until colonies were clearly visible and colonies were counted manually to estimate the survival rate. The survival rate was calculated by dividing the number of colonies formed on the arabinose-containing plate by the number of colonies on the kanamycin only plate, after accounting for dilution factors.

RESULTS

Design and construction of a selection system for homing endonuclease activity

Our selection system involves two plasmids, which link a DNA cleavage event with *E.coli* cell survival. The reporter plasmid, p11-ccdB-wtx1 (Figure 1), encodes the toxin gene *ccdB* under the inducible *BAD* promoter, followed by one copy of the endonuclease cleavage site. The *ccdB* gene was identified in F-plasmid (17) and encodes a small protein with 101 amino acids. CcdB poisons DNA gyrase and is responsible for the killing of F-plasmid free segregants during cell division (17,18). A second plasmid, pTrc-ISceI (Figure 1), contains homing endonuclease I-SceI under the *Trc* promoter. I-SceI expression is inducible by IPTG. The cleavage of reporter plasmid by I-SceI linearizes the reporter plasmid and causes it to be quickly degraded inside an *E.coli* cell by RecA (19). Thus, the expression of *ccdB* is eliminated before induction by arabinose, resulting in cell survival.
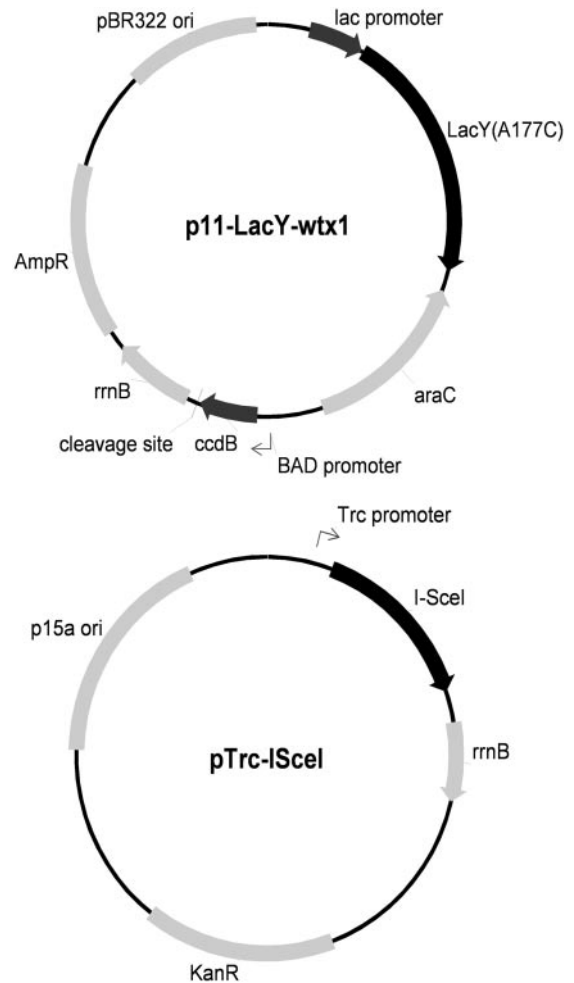
Figure 1. The two plasmids used in our *in vivo* selection system. The reporter plasmid p11-LacY-wtx1 encodes the toxin CcdB gene under the inducible *BAD* promoter and a single copy of the endonuclease cleavage site which is readily extendable to multiple copies. It also encodes an arabinose transporter gene *lacY(A177C)* under the *lac* promoter to facilitate the induction of *ccdB* by arabinose. Plasmid map of p11-ccdB-wtx1 is similar to that of p11-LacY-wtx1, except that it lacks the *lac* promoter and *lacY(A177C)* gene. Plasmid pTrc-ISceI contains homing endonuclease I-SceI under the *Trc* promoter.

One of the key criteria of this system is the tight regulation of *ccdB* gene expression so that it only exerts its toxic effect upon arabinose induction. We chose the *BAD* promoter because of its high induction ratio and tight regulation by arabinose (10). The *ccdB* gene was initially cloned into pBAD18 with a RBS—GGAGTG—borrowed from pBAD18s (10) to form pBAD-ccdB. pBAD18 plasmid has the pBR322 origin of replication and maintains 100–300 copies per cell. Transformation of pBAD-ccdB into *E.coli* BW25141 resulted in much slower cell growth in LB media containing 100 µg/ml ampicillin and low cell survival rate on the agar plate, indicating that the low level of *ccdB* expression even under the *BAD* promoter is still toxic to *E.coli* cells. To further decrease the intracellular CcdB protein concentration, we decided to decrease the level of *ccdB* translation by modifying its RBS. Using the RBS strength engineering method described in Materials and Methods section, mutant p11-ccdB, which displayed normal cell growth on LB plus 100 µg/ml ampicillin plate and no cell growth on LB plus 100 µg/ml ampicillin plus
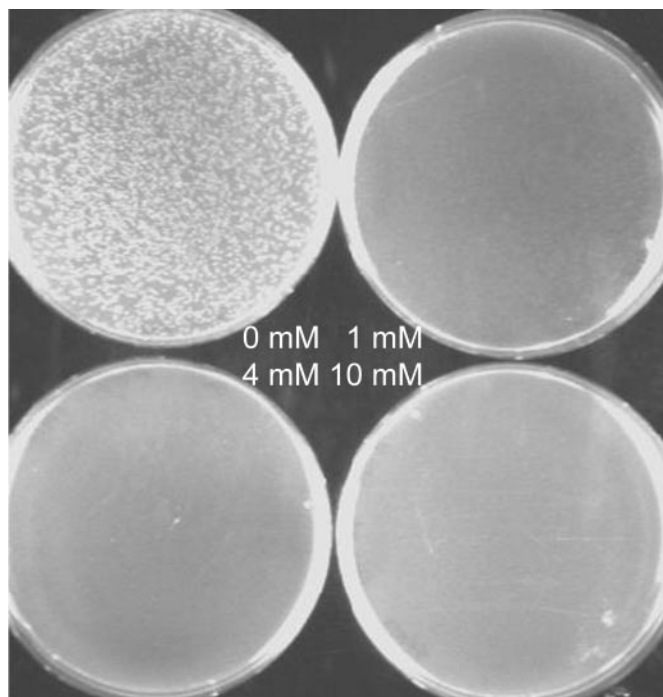
Figure 2. *E.coli* BW25141 was transformed with p11-ccdB-wtx1 and equal amount of cells were plated on agar plates containing 100 μg/ml ampicillin and 0, 1, 4 or 10 mM arabinose. These cells can form healthy colonies on ampicillin plates but lack the ability to form colonies on 4 mM arabinose-containing plates, suggesting that our engineered S.D. sequence successfully decreases the basal *ccdB* expression level but does not impair the induced *ccdB* expression level.

4 mM arabinose, was selected (Figure 2). DNA sequencing of this clone revealed its S.D. sequence to be GATTGA. One copy of the original I-SceI recognition sequence was then inserted after *ccdB* in p11-ccdB to form the reporter plasmid p11-ccdB-wtx1 (Figure 1).

The second plasmid, pTrc-ISceI encodes homing endonuclease I-SceI under the inducible *Trc* promoter. It has a p15a origin of replication and is maintained inside an *E.coli* cell with ∼15 copies. I-SceI was the first discovered homing endonuclease (20,21) and it belongs to the LAGLIDADG family. It recognizes an asymmetric DNA sequence of 18 bp 5′-TAGG-GATAA↓CAGGGTAAT-3′ and cleaves its target in the presence of Mg^{2+} to generate a 4 nt 3′ overhang (22). We used I-SceI to demonstrate the linkage between DNA cleavage and cell survival. The selection strain harboring the reporter plasmid (p11-ccdB-wtx1) was transformed with either the plasmid containing homing endonuclease (pTrc-ISceI) or control plasmid (pTrc-p15a) and subjected to the *in vivo* activity assay. All results are summarized in Table 1. Transformation with pTrc-ISceI resulted in a survival rate of 80–100%, whereas transformation with pTrc-p15a plasmid resulted in a survival rate of only 0.3–0.9%. As an additional control, the active site residue Asp44 was mutated to Ala to generate an inactive I-SceI variant D44A (23). pTrc-D44A has a survival rate of 0.5–0.9%, similar to that of pTrc-p15a. We also modified the recognition site of I-SceI to 5′-TAG GGA TAA GAa GGT AAT-3′ (p11-mISceI, a is the single base mutation), which should not be cleaved by I-SceI (22). Transformation of the selection strain containing this mutant recognition site

Table 1. Summary of the *in vivo* activity

Selection strains[a]	Survival rate[b] pTrc-IsceI	pTrc-p15a	pTrc-D44A
p11-wtx1	80–100%	0.3–0.9	0.5–0.9%
p11-LacY-wtx1	80–100%	<0.003%	ND[c]
p11-mISceI	<0.02%	ND	ND

[a]Selection strains were obtained by transforming BW25141 with plasmid p11-wtx1, p11-LacY-wtx1, and p11-mISceI and made electrocompetent.
[b]The appropriate selection strains were transformed with plasmids pTrc-ISceI, pTrc-p15a and pTrc-D44A, and aliquots were plated on LB plus 50 μg/ml kanamycin with or without 10 mM arabinose. The survival rate was calculated by dividing the number of colonies formed on the arabinose-containing plate by the number of colonies on the kanamycin only plate, after accounting for dilution factors.
[c]ND, not determined.

(p11-mISceI) with pTrc-ISceI resulted in a survival rate of <0.02%. These results indicate that our *in vivo* selection assay can efficiently link the DNA cleavage event to the cell survival through the activity of I-SceI and DNA sequence specificity.

Of note, based on previous studies (8), we thought the sensitivity of this *in vivo* selection assay may be further improved by increasing the concentration of substrate DNA sites. However, introduction of two tandem copies of I-SceI recognition site to the reporter plasmid did not yield significantly higher survival rate (data not shown), which may be owing to the very high survival rate of cells harboring only one copy of the I-SceI recognition site (80–100%) in our system.

Optimization of a selection system for homing endonuclease activity

Transformation of the selection strain harboring p11-ccdB-wtx1 with control plasmid (pTrc-p15a) resulted in a 0.3 to 0.9% cell survival rate, and longer recovery time at 37°C shaker after electroporation resulted in even higher background survival rate. Although such background survival can be greatly reduced by shortening the posttransformation recovery time in liquid SOC at 37°C, transformation efficiency and the survival rate of the selection strain transformed with pTrc-ISceI also decreases. This background survival is probably owing to the partial or complete loss of the reporter plasmid in the absence of antibiotic selection pressure (ampicillin) during recovery in liquid SOC media and on selection plates containing only kanamycin and arabinose.

The *BAD* promoter is known to be subject to all-or-none induction, because genes encoding the arabinose transporters (*araE* and *araFGH*) are also under the regulation of the *BAD* promoter (24). This autocatalytic mechanism causes only a fraction of the cells in the population to be fully induced while the remaining cells stay uninduced for an extended time period (25). We suspect this mechanism may also delay the overall induction from the *BAD* promoter. To overcome this problem, we introduced a mutant *lacY (A177C)* gene as an additional arabinose transporter (11). The *lacY(A177C)* gene was placed under the *lac* promoter on the reporter plasmid to form p11-LacY-wtx1 (Figure 1). Selection strains harboring this plasmid were transformed with pTrc-p15a or pTrc-ISceI and subject to an *in vivo* activity assay. The survival rate of pTrc-p15a cells was <0.003% (Table 1), 100- to 300-fold lower than that of the

selection strain containing p11-ccdB-wtx1, and the same high survival rate of 80–100% (Table 1) was obtained from the transformation with pTrc-ISceI.

To evaluate the efficiency of this system for identifying active homing endonuclease variants, selection strain harboring p11-LacY-wtx1 was transformed with a plasmid mixture containing a $1:10^4$ molar ratio of pTrc-ISceI : pTrc-D44A and subject to the *in vivo* activity assay. A total of 1.2×10^6 clones were screened and 60 colonies were observed on LB plus 50 μg/ml kanamycin plus 10 mM arabinose plates. Four random clones were picked and DNA sequenced, and two of the selected clones contained pTrc-ISceI, suggesting a 5000-fold enrichment. All colonies formed on the selection plates were pooled together and grown in LB plus 50 μg/ml kanamycin media overnight. Their plasmids were isolated, re-transformed into the same selection strain and subjected to *in vivo* activity assay, and an aliquot was plated on LB plus 50 μg/ml kanamycin plus 10 mM arabinose. Four random clones from the selection plate were subjected to DNA sequencing, and as expected, all four clones contained pTrc-ISceI. This assay suggests that the extremely rare functional clones from the library can be readily identified using our *in vivo* selection system, and thus we consider this new system to be suitable for directed evolution experiments.

DISCUSSION

In this work we have developed a highly sensitive *in vivo* selection method suitable for directed evolution of homing endonucleases with altered sequence specificity. This method involves the coexpression of a toxin, CcdB, and a homing endonuclease of interest, I-SceI, and couples cleavage of the plasmid expressing CcdB by I-SceI with cell survival. The simultaneous expression of the LacY(A177C) transporter protein ensures a low background survival of toxin-containing cells, thereby ensuring minimal likelihood of false positives during library screening. Our system has a number of advantages over the existing *in vivo* selection system developed by Gruen *et al.* (8).

(i) Simplicity: the mechanism of our system is more straightforward than Gruen's system but has the same or greater effectiveness. In Gruen's system, the elimination of unwanted clones requires the expression of two different proteins—mutant barnase and Amber suppressor tRNA *supE*—to kill cells. In our system, only one protein—CcdB—is required to give rise to the toxic phenotype. Furthermore, in Gruen's system, it is necessary to express the target homing endonuclease as an N-terminal fusion protein of Amber suppressor tRNA *supE*. Such fusion may introduce unexpected and unwanted homing endonuclease—suppressor *supE* interactions. Our system does not have such complications.

(ii) Sensitivity: our system is much more responsive to the homing endonuclease cleavage event. The survival rate of cells with wild-type I-SceI and one copy of the original I-SceI recognition site is 80–100% in our system, whereas in Gruen's system, the survival rate of cells harboring I-SceI and two copies of the I-SceI recognition site is 25%. Considering that the survival rate of the selection system

can not be >100% and it was proportional to the concentration of substrate DNA sites (8), our system is much more sensitive than that developed by Gruen *et al.* (8). The high cell survival rate obtained in our system is probably owing to the higher I-SceI expression level resulting from using the *Trc* promoter instead of the *lac* promoter, and longer posttransformation recovery time at 37°C (40 or 70 min in our system versus 20 min in Gruen's system) before plating on arabinose-containing plates. By introducing an arabinose transporter gene *lacY(A177C)*, the background cell survival rate is kept under 0.003%, which makes this system feasible for directed evolution approaches.

Introduction of mutant *lacY(A177C)* is critical for the low background survival rate. The background survival of cells without I-SceI is probably owing to the complete or partial loss of reporter plasmid that causes the reduction or elimination of the toxin *ccdB* gene expression. This gradual loss of reporter plasmid should be proportional to the number of cell divisions, or the time of cell growth in media lacking the appropriate antibiotic selection pressure. After transformation, the selection strains were recovered first in liquid SOC media and then plated on arabinose-containing agar plates; in both stages no ampicillin was added to maintain the reporter plasmid. In most cases only a subpopulation of cells are immediately induced upon plating on arabinose-containing plate while the rest of the cells undergo arabinose induction much later. This is due to the autocatalytic transcription mechanism of the *BAD* promoter where a threshold of intracellular transporter concentration is required to accumulate enough inducer at the time the sugar is added. This initial accumulation results in the induction of increased expression of transporters that catalyze the uptake of more sugar, leading to further induction (25,26). This extra time required for the full induction of *BAD* promoter might be sufficient for the slow-inducing cells to lose their reporter plasmid. Thus, we introduced a recombinant arabinose transporter gene, *lacY(A177C)* (11), to (i) facilitate the homogeneous induction of *ccdB* by arabinose within the cell population, (ii) force the immediate induction by arabinose upon plating on the arabinose-containing plates, and (iii) raise the intracellular arabinose concentration and in turn increase the transcription efficiency of each individual *BAD* promoter so that cells with decreased reporter plasmid copy number can still produce enough CcdB to kill the cell. A modified *in vivo* activity assay, with extended posttransformation recovery time at 37°C and an additional incubation at 30°C, was necessary for the selection strain containing p11-LacY-wtx1 to have a high (80–100%) survival rate with pTrc-ISceI. This modified *in vivo* activity assay does not have significant effect on the background cell survival rate.

The success of our system heavily relies on the tight regulation of toxin *ccdB* expression. The *BAD* promoter has rapid kinetics (27), a high induction ratio of up to 1200-fold (10) and tight control (28), and was thus chosen as the promoter for *ccdB*. However our initial effort of expressing ccdB from an existing *BAD* promoter still suffered relatively high basal level of *ccdB* expression that resulted in a low survival rate in the absence of arabinose. Thus, further engineering was necessary to decrease this basal level expression while maintaining the relative high expression level upon induction. We first

attempted to use a ΔcyaA E.coli strain because the BAD promoter is catabolite sensitive (29). However cells lacking this essential cyaA gene show severely retarded growth, as was observed in other studies (14). Subsequent efforts focused on methods such as modifying mRNA stability via RNA hairpins (9) and verifying plasmid copy number (30). We decided the simplest way to achieve our goal was to modify the S.D. sequence. This method has been applied previously to obtain a higher protein expression level (31), but has never been used for the purpose of decreasing the target protein expression level. As shown in our study, RBS engineering is indeed an extremely simple and effective way to modify protein expression levels, as we observed a wide range of protein expression levels from our library screening. This strategy may be useful for the over-expression and purification of toxic proteins when a low basal expression level is necessary to maintain cell survival in the uninduced state. It may also find applications in metabolic engineering when several genes are required to be expressed simultaneously inside an E.coli cell to different degrees. By assigning a different S.D. sequence to each individual target gene, these genes can be differentially expressed from a common promoter. In fact, this mechanism has been widely used by nature when an operon is regulated by a single promoter and the members of the operon are expressed at various levels according to their own S.D. sequence.

The survival rate of pTrc-ISceI in the selection strain containing a modified I-SceI recognition site (p11-mISceI) (<0.02%) is much lower than that of pTrc-p15a in the strain containing the original I-SceI recognition site (p11-ccdB-wtx1) (0.3–0.9%). As discussed previously, the background cell survival is probably owing to the partial or complete loss of the reporter plasmid, which should correlate with the number of cell divisions. Cell division can be influenced by many factors, such as the metabolic burden encountered by each cell. We reason that the lower survival rate of pTrc-ISceI may be owing to the induced expression of I-SceI, which consumes cellular energy and slows the cell cycle of division. In the case of pTrc-p15a, more cellular resources can be used for cell growth and division.

As a test run, wild-type I-SceI and mutant D44A were mixed at a molar ratio of $1:10^4$ and transformed into selection strain containing p11-LacY-wtx1. It was demonstrated that the 0.01% wild-type I-SceI in the library can be readily identified using our in vivo activity assay.

In principle, this system can also be modified to include a negative selection to select for homing endonuclease mutants that display a specificity shift. This can be readily achieved by incorporating the original recognition sequence to the homing endonuclease encoding plasmid (pTrc-ISceI). Transformation of the reporter strain with variants that retain significant cleavage activity toward the original sequence results in the loss of these variants encoding plasmids. As a result, only variants with a shifted specificity will be selected from the library of variants created by directed evolution approaches.

We are currently applying this method for the engineering of I-SceI variants with altered sequence specificity. So far, we have screened a library of 2×10^6 I-SceI variants using this system and we have successfully identified a few variants that showed significant activity towards the new DNA substrate (TAGGGATAACAGGGCCTA) (data not shown). In principle, this in vivo selection system may be used to assay the activity and perform directed evolution of other DNA cleavage enzymes.

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